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2 South Parks Road
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Patents ADP number *(if you know it)* 39985640001

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4. Title of the invention — HLA-E BINDING

5. Name of your agent *(if you have one)*

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HLA-E BINDING

This invention relates to methods of identifying, targeting and of isolating a group of CD94+ cells, in particular a group of CD94+ cells including natural killer (NK) cells and a subset of T cells. The invention also relates to methods of targeting functional moieties such as toxins to the CD94+ cells. The invention further relates to multimeric complexes of HLA-E for use in the methods.

Human leukocyte antigen-E (HLA-E) is a nonclassical MHC class Ib molecule of very limited polymorphism. Human nonclassical MHC class Ib molecules (which include HLA-E, HLA-F and HLA-G) are homologous to classical MHC class Ia molecules (HLA-A, HLA-B and HLA-C) but are characterised by limited polymorphism and low cell surface expression (reviewed in Shawar et al 1994 *Annu. Rev. Immunol.* 12: 839). The mouse MHC class Ib molecule Qa-1 shares some characteristics with HLA-E in that it displays a broad tissue distribution and some structural similarities in the peptide-binding groove (reviewed in Soloski et al 1995 *Immunol. Rev.* 147: 67).

Whereas the function of the classical MHC class I molecules in presenting peptides derived from cytosolic proteins to CD8+ T cells is well-established, the function of nonclassical MHC molecules remains unknown, in particular for HLA-E. HLA-E is transcribed in most tissues. Recently, we have shown that HLA-E is able to bind in its peptide-binding site peptides derived from MHC class I leader sequences at positions 3 to 11 (Braud et al 1997 *Eur. J. Immunol.* 27: 1164-1169). The optimum binding peptide is a nonamer. Using alanine and glycine substitutions, it was established that there are primary anchor residues at positions 2 and 9 of the peptide and secondary anchor residues at position 7 and possibly position 3. The literature suggests that HLA-E is localised in the endoplasmic reticulum (ER) and might have a role in the loading of

peptides onto classical MHC class I molecules in a similar way to HLA-DM for MHC class II molecules. Neither mouse cells transfected with HLA-E and human β2microglobulin (β2m) nor the 721.221 cell line which only expresses HLA-E and HLA-F, show surface expression of HLA-E (Ulbrecht et al, *J. Immunol. 1992* 149: 2945 - 2953 and *J. Exp. Med. 1992* 176: 1083 - 1090).

Assembly of MHC class I molecules occurs in the endoplasmic reticulum (ER) and requires peptide translocation through the transporter associated with antigen processing (TAP) (reviewed in Cerundolo et al 1996 In *HLA and MHC genes, molecules and function,* Edited by Browning M, McMichael A. Oxford: Bios. Scientific Publisher Ltd; 193 - 223). In human cells, newly synthesised MHC class I heavy chains associate with calnexin which is later displaced by the association of β2m. Following dissociation of calnexin, class I-β2m heterodimers are stably associated with another ER resident protein, calreticulin. Another molecule, tapasin, which is associated with TAP and with MHC class I-calreticulin complexes, acts as a bridge between them. MHC class I association with TAP facilitates peptide binding and the class I molecules are released and exported to the cell surface upon stable loading of peptide.

Natural killer (NK) cells are cytotoxic cells which are normally defined by their activity. NK cells have the morphology of large granular lymphocytes. They use recognition systems which are not yet clearly understood. Recognition of tumour cell lines and virally-infected cells is however driven by the absence of MHC class I at the target cell surface (some MHC class I molecules interact with specific NK receptors). NK cell cytotoxicity is mediated by the interaction between Fas and Fas ligand or by the release of the contents of the intracellular granules including the pore forming protein perforin and the serine protease granzyme B. NK cells are generally but not exclusively CD3- and CD56+. They may also be

CD16+ and some are also CD8+. Certain CD8+ T cells have an NK cell-like function, in that they are able to kill MHC class I negative cells.

It has now been discovered that HLA-E is stably expressed at a low level on the surface of cells. Its expression at the surface correlates with co-expression of human MHC class I molecules which possess a peptide in their leader sequence capable of binding to HLA-E. Loading of these signal sequence-derived peptides is TAP and Tapasin-dependent and HLA-E assembly appears to be similar to classical MHC class I assembly (Braud et al 1998 *Current Biology* 8).

It has also been discovered that HLA-E binds to NK cells expressing receptors CD94/NKG2A, B and C. The majority of NK cells express CD94 and NKG2 and the majority of NK cells are capable of binding HLA-E. HLA-E also binds to a small subset of T cells expressing CD94 and NKG2.

Furthermore, it has been discovered that polymeric HLA-E molecules bind strongly to the NK cells and T cell subset.

In its broadest sense, the invention provides the use of the interaction between HLA-E and NK cells and/or a subset of T cells, to identify and/or target and/or isolate those cells; and HLA-E in a suitable form for such use.

The invention provides in one aspect a method of identifying the presence of CD94+/NKG2+ NK cells and T cells in a sample, which method comprises contacting the sample with HLA-E under suitable binding conditions and detecting binding of HLA-E to the cells.

In another aspect, the invention provides a method of selecting NK cells and a subset of T cells from a sample, which method comprises contacting the sample with HLA-E under binding conditions and separating cells bound to the HLA-E from the mixture.

In another aspect, the invention provides a multimer of HLA-E having enhanced binding capability compared to non-multimeric

HLA-E, optionally labelled with a detectable label.

In another aspect, the invention provides a method of killing or inactivating NK cells and a subset of T cells, which method comprises contacting the cells with HLA-E under binding conditions and carrying out targeted killing on the bound cells. Any targeted killing method may be used, for example NK cells may be identified by detecting bound HLA-E, and then destroyed by use of a laser.

In further aspects the invention provides HLA-E coupled to a toxic agent and a method of killing or inactivating NK cells and a subset of T cells using the coupled HLA-E.

Preferably, the methods according to the invention employ recombinant HLA-E in the form of a multimeric HLA-E molecule. The preferred multimer is a tetramer, but other multimers for example dimers, trimers and multimers containing 5, 6, 7 etc. HLA molecules are not excluded.

The invention is useful for diagnostic purposes. Detection and/or quantitation of NK cells or a sub-population of NK cells, or a sub-population of T cells (which may be further identified by co-staining with anti-CD8 or anti-CD4 antibody or antibodies or ligands to other T cell markers) will be useful in a variety of conditions, including the following:

(i) <u>Cancer, Lymphomas and Leukaemias (particularly large granular cell Leukaemias).</u>

NK cells are believed to have an anti-tumour cell activity. A marker for progress of therapy, or simple prognosis, can be provided by monitoring NK cell numbers and state of activation. The state of activation can be investigated by co-staining with antibodies to activation markers. This will provide an extremely simple test.

(ii) <u>Infections</u>

NK cell numbers may change during viral or other infections

and knowing their numbers could be of great value for example in HIV infected patients. It will be of particular interest to monitor NK cell numbers in cytomegalovirus (CMV) infections. CMV has sequences in its proteins that are capable of affecting HLA-E expression.

(iii) Pregnancy

There is interest in the role of NK cells in the placenta, in the prevention of rejection of the foetus. The invention provides a means to monitor NK cells in the placenta.

(iv) Transplantation

NK cells may be involved in transplant rejection and graft-versus-host disease (GVHD) after bone marrow transplantation. Monitoring of NK cells may be of value in patient management.

(v) Immunodeficiency

The diagnostic use of HLA-E can be extended to the detection of new immunodeficiency syndromes, either inherited or acquired, which exhibit lower or higher than normal NK cell levels. Some treatments may be toxic or stimulatory to NK cells.

(vi) Autoimmune Diseases

It will be useful to monitor NK cells in autoimmune diseases such as systemic lupus erythenatosus, diabetes, thyroid diseases, vitiligo, rheumatoid arthritis etc.

(vii) Following Treatment

The invention also enables the monitoring of secondary effects of any treatment which could lead to up or downregulation of CD94/NKG2+ NK cells.

Although NK cells are specifically referred to in the examples of diseases and conditions above, monitoring of the T cell subset expressing receptors recognised by HLA-E is also included.

The invention also has a range of therapeutic applications.

Examples include the following:

(i) Enhancing NK Levels

The invention provides a method of selecting HLA-E binding NK cells or T cells from a mixed cell population. The selected cells can be expanded *in vitro* and returned to the patient. Such treatment may be effective in some serious infections or cancers where a growth deficiency of these cells is associated with poor prognosis.

(ii) Removing NK Cells

The invention provides methods and means for removal of HLA-E binding NK cells and T cells, for example from bone marrow to be used as donor bone marrow in transplantation. T cell depletion of bone marrow is already known. HLA-E coupled to a toxin could be used to destroy HLA-E binding cells *in vivo*.

Described herein in detail is a method of making HLA-E tetramers by biotinylating recombinant HLA-E and constructing complexes by refolding the HLA-E *in vitro* with β2m molecules and with a synthetic leader peptide from HLA-B. In the method described, biotinylation sites are engineered into the C terminus of the HLA-E heavy chain.

Multimers of HLA-E can be produced by alternative methods. For example, antibody cross-linking can be used to produce dimers. Other multimerisation techniques which may be employed include cross-linking techniques using cross-linking agents such as flurogenic compounds or other chemical cross-linking agents. Also, variations of the tetramerisation method described may be employed. For example, the β2m could be chemically biotinylated at its seven lysine residues, four of which are in an appropriate position to allow complexing.

Whatever the method used, a suitable peptide for binding in the HLA-E peptide groove will be required. Tables 1 and 2 show examples

of the possible peptides found at residues 3 to 11 of MHC class I leader sequences. The tables indicate which of these are found to bind to HLA-E *in vitro*. Other peptides which bind to HLA-E may be used, for example peptides from viral proteins which may be the same as or different to MHC class I leader sequence peptides. Although nonamer peptides are usually the optimum size for binding to HLA-E, it is possible that peptides which are slightly shorter or slightly longer, e.g. by one or two residues at one or both ends, will also work. A suitable assay for identifying synthetic peptides which bind to HLA-E is described in Braud et al 1997 *Eur. J. Immunol.* 27:1164-1169.

Labelling of the HLA-E may be carried out by any suitable method. Described herein is tetrameric HLA-E labelled with phycoerythrin via the extravidin used to form the tetrameric complex. A variety of other labels may be employed at a variety of possible sites on the multimer, and the HLA-E may be labelled before, during or after multimerisation. Useful labels commonly employed for labelling proteins include radioactive, fluorescent and enzymatic labels. These and other detectable labels may be employed in the invention.

HLA-E itself exhibits very little polymorphism. The sequences for two different alleles of HLA-E can be found in the following data base locations: E*0101 at M20022 (arg in the residue at position 107); E*01031 at M32507 (glycine residue at position 107).

It will be evident that in addition to any special features such as biotinylation sites required for multimerisation, the recombinant HLA-E used in the invention may have other features which make it different to native HLA-E. For example, the recombinant HLA-E may have deletions or insertions or altered residues compared to native HLA-E, which result in improved properties such as enhanced binding capability or improved stability, for use in accordance with the invention. HLA-E having improved stability at elevated temperatures, such as temperatures over 4°C and/or

over room temperature and/or at or around 37°C, will be of particular interest.

Methods of separating a population of cells from a mixture of cells are well known. For example, the isolation of T cells using rosetting procedures is well known and exploits the unique ability of T cells to bind to and form rosettes with sheep red blood cells (SRBC). CD2 on T cells is the SRBC receptor. (Weiner et al 1982 *J. Immunol. Methods* 50:3999-4049).

Some known separation methods will be adaptable for use in the separation or isolation of CD94+/NKG2+ cells using HLA-E. For example, T cell populations can be isolated by use of antibody-coated plates. The antibodies are specific for particular cell-surface markers. Cell separation can be a negative selection process or a positive selection process (Wysocki et al, 1978 *PNAS* 75:2840-2848). HLA-E coated plates may be used to separate CD94+/NKG2+ cells.

Immunomagnetic purification of a T cell subpopulation can also be realised using suitable antibodies coated on magnetic beads, in a negative or a positive selection process (Funderud et al 1987 in *Lymphocytes: A Practical Approach* Oxford University Press, New York 55-61). HLA-E-coated beads may be similarly employed for selection of CD94+/NKG2+ NK cells and T cells.

FACS (fluorescence activated cell sorting) techniques may also be employed. Cell sorting of fluorescence-labelled cells uses flow cytometry to monitor the expression of specific intracellular and cell surface molecules and sort cell populations (Fleisher et al 1988 *Cytometry* 9:309-315).

Techniques which may be used in accordance with the invention for selective depletion or targeted killing of CD94+/NKG2+ cells in a mixed cell population include antibody/complement-mediated cytotoxicity. Using a complement-fixing antibody, the cells expressing the marker

recognised by the antibody can be lysed in presence of complement (Bianco et al 1970 *J. Exp. Med.* 132:702-720). An anti-HLA-E antibody may be employed to selectively destroy cells to which HLA-E is bound.

TABLE 1

Examples of peptides generated from MHC class I leader sequences at residues 3-11

Leader sequence peptide (3-11) from MHC class I	Binding to HLA-E in vitro
-VMAPRTLVL	+
-VMAPRTLLL	+
-VMAPRTVLL	+
-VMAPRTLIL	+
-VMAPRTLFL	+
-VMGPRTLVL	+/-
-VTAPRTVLL	-
-VTAPRTLLL	-

EXAMPLES

Example 1

Construction of HLA-E tetrameric complexes

HLA-E-tetrameric complexes were constructed by refolding recombinant HLA-E and β2m molecules *in vitro* with a synthetic peptide (VMAPRTVLL) derived from residues 3-11 of the signal sequence of HLA-B*0801. A biotinylation site was engineered in the C terminus of the HLA-E heavy chain, allowing HLA-E/β2m/peptide complexes to be enzymatically biotinylated using *E.coli* BirA enzyme and conjugated with phycoerythrin (PE)-labelled Extravidin to create tetrameric complexes. HLA-A and –B tetramic complexes have proved to be very efficient at specifically binding to T cell receptors on antigen-specific $CD8^+$ T cells from peripheral blood *in vitro* (Altman *et al* 1996 *Science* 274: 94-96).

Methods

HLA-E was cloned by RT-PCR with primers COO7 and COO6 from RNA extracted from monocytes of an HLA-E*0101 homozygous individual. The N terminal nucleotide sequence was synonymously altered by PCR mutagenesis using the primers CO17 and COO6 to optimise protein expression from the pGMT7 vector in E. coli. The coding sequence for the extracellular portion of HLA-E (residues 1-276) was amplified using the primers CO17 and CO23 and recloned into a pGMT7 derivative to produce the expression plasmid COCO92 which contains the BirA recognition and biotinylation site in frame at the 3' end of the HLA-E heavy chain. Primers were:

COO6 gtgggctaagcttacggcttccatctcagggtgacgggctc

COO7 ctacgggcatatggtagatggaaccctccttttactctcc

CO17 ccgtacctcgagcatatgggttctcattctttaaaatattttcatacttctgtatctagacccggccg

CO23 tggtgtctagaggatcctggcttccatctcagggtgacgggctcg

HLA-E tetrameric complexes were generated essentially as

described (Altman *et al* 1996). Briefly, HLA-E and β2m proteins were over-expressed *in E. coli* strains BL21 (DE3) pLysS and XA90 respectively, purified from inclusion bodies, solubilised into a urea solution, then refolded by dilution *in vitro* with a synthetic peptide (VMAPRTVLL) from HLA-B*0801 leader sequence (Research Genetics). HLA-E heavy chain/β2m/peptide complexes were biotinylated with BirA enzyme, purified by FPLC and Mono-Q ion exchange chromatography, then complexed in a 4:1 molar ratio with Extravidin-PE (Sigma).

Example 2

Binding of HLA-E tetramers

Peripheral blood mononuclear cells (PBMC) from 9 normal donors were stained with HLA-E tetramer prepared and described in Example 1 and compared to staining observed with an HLA-A2 tetramer refolded with Epstein Barr Virus (EBV) lytic cycle BMLF1 259-267 peptide epitope (Steven *et al* 1997 *J.Exp. Med.* 185: 1605-17). A high frequency of lymphoid cells were stained with the HLA-E tetramer (range 2 to 11%) (Fig. 1A), whereas the HLA-A2 tetramer generally stained 0 to 0.8% of the lymphocytes in EBV-seropositive donors (Fig. 1C). By setting an electronic gate on the lymphocytes binding HLA-E tetramer, we observed that a large proportion were NK cells (typically 40 to 80 % $CD3^-$, $CD56^+$) but a significant subset were T cells (typically 15 to 50% $CD3^+$), some of which were also expressing CD56 (Fig. 1B). About 2% of the lymphocytes binding HLA-E tetramer were $CD4^+$ T cells, and about 5 % were $CD19^+$ B cells, but these could represent non-specific binding because of similar staining with the HLA-A2 tetramer (data not shown). The HLA-A2 tetramer did not bind to $CD56^+$ cells but, in EBV-seropositive donors, bound to EBV specific $CD3^+$, $CD8^+$ T cells (Fig. 1D), confirming previous studies on the specificity of MHC-tetrameric complexes for T cells bearing a specific T cell receptor Altman *et al* 1996).

HLA-E tetramer staining was abolished when the PBMC and the tetramer were incubated in the presence of the antibody HP3D9 (Aramburu *et al* 1990 *Immunol.* 144: 3238-47) against CD94, an NK cell receptor belonging to the C-type lectin superfamily (Chang *et al* 1995 *Eur J. Immunol.* 25: 2433-37) (Fig. 2A). As the antibody HP3D9 was diluted, the HLA-E tetramer staining was restored (data not shown). The interaction between HLA-E and CD94 was also confirmed by staining a number of well-characterised $CD94^+$ NK clones with HLA-E tetramer and demonstrating that another anti-CD94 mAb (DX22) (Phillips *et al* 1996 *Immunity* 5: 163-172) completely inhibited HLA-E tetramer binding (Fig. 2B, and data not shown). No staining with HLA-A2 tetramer was found on $CD94^+$ NK clones (data not shown).

To characterise further the NK receptor interacting with HLA-E, we stained P815 and 293T cells transfected with these receptors. No HLA-E tetramer staining was observed on P815 stably transfected with CD94 alone or NKG2B alone (Fig. 3A), nor on 293T transiently transfected with CD94 or NKG2A alone (data not shown). In contrast, HLA-E tetramer bound to 293T cells cotransfected with CD94 and NKG2A, CD94 and NKG2B, or CD94 and NKG2C (Fig. 3B). Expression of the heterodimers on these transfectants was monitored using a polyclonal rabbit serum that reacts with CD94/NKG2A, NKG2B and NKG2C heterodimers (Lazetic *et al* 1996 *J. Immunol.* 157: 4741-45). This result was confirmed using mouse pre-B Ba/F3 cells stably transfected with CD94/NKG2C or NK clones expressing the inhibitory receptor CD94/NKG2A (Fig 2B and data not shown). Carbohydrates on HLA-E are not necessary for binding, as the recombinant HLA-E used to make the tetramer was produced in *E. coli.* This is quite surprising given that both CD94 and NKG2 proteins are members of the C-type lectin superfamily. Carbohydrate residues may form additional points of interaction increasing the affinity of binding. Recombinant HLA-E produced in *E. coli* did not have sufficient affinity to

bind and stain NK cells when incubated as single refolded HLA-E-β2m-peptide molecules (data not shown).

We have also shown that HLA-E does not interact with other killer cell inhibitory cell receptors (KIR) as no staining with the HLA-E tetramer was observed on Ba/F3 cells transfected with KIR2DL1 (NKAT1 or p58), KIR2DL3 (NKAT2 or p58), KIR3DL1(NKAT3 or p70), KIR3DL2 (NKAT4 or p70/140), KIR2DS2 (NKAT5 or p50), KIR2DL2 (NKAT6 or p58) or KIR2DS4 (NKAT8 or p50) (Lanier *et al* 1997 *Immunol. Rev.* 155: 145-154). Furthermore, staining of PBMC with HLA-E tetramer was not blocked by antibodies against any of these KIR receptors: EB6 (anti-KIR2DL1), GL183 (anti-KIR2DL3, -KIR2DS2, -KIR2DL2), DX9 (anti-KIR3DL1), or 5.133 (anti-KIR3DL1, -KIR3DL2) (data not shown). Thus, the CD94/NKG2 receptors appear to be unique and specific receptors for HLA-E recognition.

We have previously reported that HLA-E, like the mouse Qa-1 molecule (Aldrich *et al* 1994 *Cell* 79: 649-658; DeCloux *et al* 1997 *J. Immunol.* 158: 2183-2191; Cotterill *et al* 1997 *Eur. J. Immunol.* 27: 2123-2132) can bind signal sequence-derived peptides from MHC class I molecules in vitro and recently showed that HLA-E cell surface expression is regulated by the binding of such peptides (Braud *et al* 1997). Most HLA-A and HLA-C alleles possess a leader peptide 3-11 that binds to HLA-E whereas only a third of HLA-B alleles do. The remaining B alleles have a Threonine at position 2 in the peptide instead of a Methionine. This substitution at a primary anchor residue disrupted peptide binding to HLA-E as measured in an *in vitro* peptide binding assay previously described (Braud *et al* 1997) (Table 2). Transfection of MHC class I alleles which have a leader peptide capable of binding to HLA-E into HLA-A, -B, -C, -G negative 721.221 cells resulted in expression of the endogenous HLA-E on the cell surface of 721.221. When the leader sequence peptide was not capable of binding, no such upregulation of HLA-E was observed.

It has been shown previously that NK cells expressing an inhibitory CD94/NKG2A receptor do not kill 721.221 cells transfected with certain HLA-A, -B, -C, or –G alleles, but are able to lyse these transfectants in the presence of neutralising anti-CD94 or anti class I antibodies (Phillips *et al* 1996 *Immunity* 5: 163-172; Sivori *et al* 1996 *Eur. J. Immunol.* 26:2487-2492; Sivori *et al* 1996 *Transplant* 28: 3199-3203). A striking correlation between the presence of an HLA class I leader sequence peptide capable of binding to HLA-E causing its surface expression and the specificity of the CD94/NKG2A inhibitory receptor is shown in Table 2. All the MHC class I alleles which, upon transfection, protect 721.221 cells from killing by CD94/NKG2A+ NK clones have a peptide capable of binding to HLA-E. Similarly, all HLA alleles incapable of protecting against these clones lack an HLA-E binding leader peptide. Together with the direct evidence for physical interaction between HLA-E and CD94/NKG2A, these results imply that inhibition by the CD94/NKG2A receptor is mediated by recognition of HLA-E rather than a broad range of HLA-A, -B, and –C molecules. In support of this, the HLA-A2 tetramer refolded around a Tax peptide epitope of human T-cell lymphotropic virus HTLV1 (Garboczi *et al* 1996 *Nature* 384: 134-141) did not bind to CD94/NKG2A transfectants or NK cells expressing CD94/NKG2 receptors, despite the fact that HLA-A2 has been shown to have a protective effect against CD94/NKG2A+ NK clones and HLA-A2 target cell protection can be reversed in the presence of anti-CD94 or anti-class I antibodies. However, a role for HLA-A2 cannot be totally excluded if the interaction between MHC class I and CD94/NKG2A receptor requires specific peptides.

It has recently been demonstrated that recognition of 721.221 target cells by CD94/NKG2A$^+$ NK clones can be inhibited by transfection of HLA-G, another nonclassical class I molecule mainly expressed on trophoblast cells (Soderstrom *et al* 1997 *J. Immunol.* 159: 1072-1075; Perez Villar *et al* 1997*J. Immunol.* 158: 5736-5743; Pende *et al* 1997 *Eur.*

J. Immunol. 27: 1875-1880). However, HLA-G also possesses a leader sequence peptide capable of binding to HLA-E and 721.221-G transfectants express a significant level of HLA-E. Similarly, Reyburn *et al* 1997 (*Nature* 386: 514-517) recently reported that human cytomegalovirus encodes a viral protein (UL18), with similarity to MHC class I, that can protect 721.221 cells from NK cell lysis, possibly involving CD94 receptors. Whether these observations can be explained by binding of HLA-G or UL18 leader peptides to the endogenous HLA-E molecules in 721.221 is under investigation.

HLA-E also binds to CD94/NKG2C which has been shown to activate cytolytic activity in NK cell transfectants (Houchins *et al* 1997 *J. Immunol.* 158: 3603-3609) indicating that HLA-E is involved in regulating NK cell-mediated cytotoxicity via both CD94/NKG2A inhibitory and CD94/NKG2C stimulatory NK cell receptors. Our present results demonstrate a novel role for a nonclassical class I molecule HLA-E and identify its predominant receptor. It remains to be determined whether the strong preference of HLA-E for binding signal sequence-derived peptides is simply to permit expression of HLA-E or whether it is implicit in recognition by CD94/NKG2 receptors.

Table 2

.221 cells tranfected with HLA class I alleles	Inhibition of killing by CD94/NKG2A + NK clones*	Presence of leader sequence peptide capable of binding to HLA-E§	HLA leader sequence peptide (residues 3-11)	Concentration of peptide required to obtain 50% of binding to HLA-E†
.221	-	- ‡		
.221-A*0201	+	+	VMAPRTLVL	0.06 μM
.221-A*0211	+	+	VMAPRTLVL	0.06 μM
.221-A*2501	+	+	VMAPRTLVL	0.06 μM
.221-A*2403	+	+	VMAPRTLVL	0.06 μM
.221-A*3601	+	+	VMAPRTLLL	0.3 μM
.221-B*0702	+	+	VMAPRTVLL	0.06 μM
.221-Cw*0102	+	+	VMAPRTLIL	0.3 μM
.221-Cw*0401	+	+	VMAPRTLIL	0.3 μM
.221-Cw*0304	+	+	VMAPRTLIL	0.3 μM
.221-Cw*0801	+	+	VMAPRTLIL	0.3 μM
.221-G	+	+	VMAPRTLFL	0.3 μM
.221-B*1501	-	-	VTAPRTVLL	> 100 μM
.221-B*5101	-	-	VTAPRTVLL	> 100 μM
.221-B*5801	-	-	VTAPRTVLL	> 100 μM
.221-B*4601	-	-	VTAPRTVLL	> 100 μM
.221-B*5401	-	-	VTAPRTLLL	> 100 μM
.221-B*5501	-	-	VTAPRTLLL	> 100 μM

*Results published by Phillips *et al* 1996

† A peptide binding assay was developed in vitro. Results are expressed as a ratio of optical densities referred to as percentage of binding to HLA-E (Braud *et al* 1997)

‡ The HLA-A, -B, -C, and –G negative .221 cells express HLA-E and HLA-F which have a shorter leader sequence and lack the appropriate peptide capable of binding to HLA-E.

§ The presence of a leader sequence peptide capable of binding to HLA-E upregulates HLA-E surface expression as measured on .221 and

.221 cells transfected with HLA-A or –B alleles using the antibody DT9 recognizing HLA-E and HLA-C alleles

Figure Legends

Figure 1 HLA-E tetramer binds NK cells and a subset of T cells

Flow cytometry analysis on gated peripheral blood lymphocytes from normal EBV seropositive donor VB using (A) HLA-E tetramer refolded around the leader sequence peptide residues 3-11 from HLA-B*0801 or (C) HLA-A2 tetramer refolded around the Epstein Barr Virus (EBV) lytic cycle BMLF1 259-267 peptide epitope (Steven *et al* 1997). The phenotypes of (B) HLA-E tetramer or (D) HLA-A2 tetramer binding lymphocytes were further investigated in triple colour stains as indicated. Percentages in each quadrant are represented by the cross in the upper right. Within the total CD3-, CD56+ NK cell population, 10.3 % of cells bound HLA-E tetramer, and within the total CD3+ T cell population, 2.2 % of cells bound HLA-E tetramer. In contrast, less than 0.2 % of CD3-, CD56+ cells bound HLA-A2 tetramer, whereas 1 % of CD3+ T cells bound HLA-A2 tetramer.

Figure 2 HLA-E tetramer staining is inhibited by anti-CD94 antibodies

(A) Peripheral blood lymphocytes from normal donor SRJ were stained with the anti-CD94 antibody HP3D9 (Aramburu *et al* 1990) (1/50 dilution of ascites) followed by FITC-anti-mouse IgG $(Fab')_2$ (Sigma); HLA-E tetramer-PE alone; or HLA-E tetramer-PE in the presence of HP3D9 (1/50) which inhibited HLA-E tetramer staining.

(B) The NK cell line NKL (Robertson *et al* 1996 *Exp. Haematol.* 24: 406-415) expressing the NK receptor CD94/NKG2A but none of the KIR molecules was stained with the anti-CD94 antibody DX22 (Phillips *et al* 1996) (1mg) followed by PE-anti-mouse IgG; HLA-E tetramer-PE; or HLA-

E tetramer-PE in the presence of 1 mg of DX22 antibody which inhibited HLA-E tetramer staining. Percentages in each quadrant are listed in the upper right. The HLA-A2 tetramer refolded around the HTLV1 Tax peptide (Garbocz *et al* 1996) did not bind to NKL (data not shown).

Figure 3 HLA-E binds to NK cell CD94/NKG2A, CD94/NKG2B and CD94/NKG2C receptors but not to CD94 or NKG2 alone.

(A) P815 cells were stably transfected with pBJ-neo vector containing human CD94 cDNA (Chang *et al* 1995) or NKG2B cDNA (Houchins *et al* 1991 *J. Exp. Med.* 173: 1017-20). Cells were stained with PE-control mouse IgG1 (cMIgG1) or IgG2b (cMIgG2b), anti-CD94 antibody DX22-PE, anti-NKG2A and B antibody DX20-PE, or HLA-E tetramer-PE. Neither P815 transfectant stained with HLA-E tetramer or HLA-A2 HTLV1 Tax peptide tetramer. (B) 293T cells stably transfected with CD94 were transiently transfected with NKG2A, NKG2B, and NKG2C (Lazetic *et al* 1996). Flow cytometry staining was performed using rabbit preimmune serum (cRIgG) 1/500 final dilution or rabbit anti-CD94/NKG2 heterodimer serum (anti-CD94/NKG2) 1/500 final dilution, both followed by FITC- anti-rabbit IgG, or with HLA-E tetramer-PE. No staining with HLA-A2-HTLV1 tax peptide tetramer was observed. Staining of 293T-CD94 cells cotransfected with a control plasmid were not stained by HLA-E tetramer or the rabbit anti-CD94/NKG2 serum (data not shown).

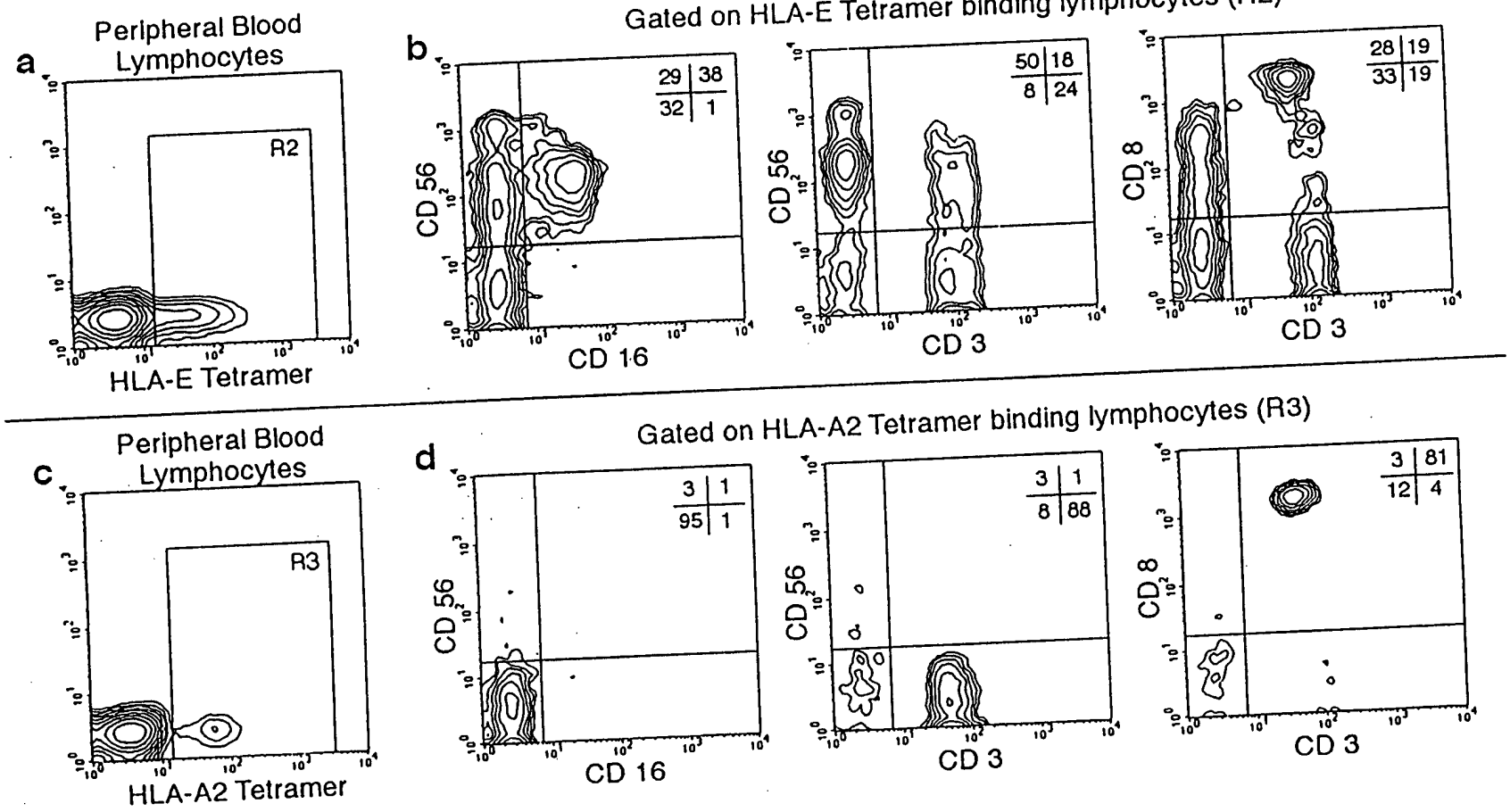

Figure 1

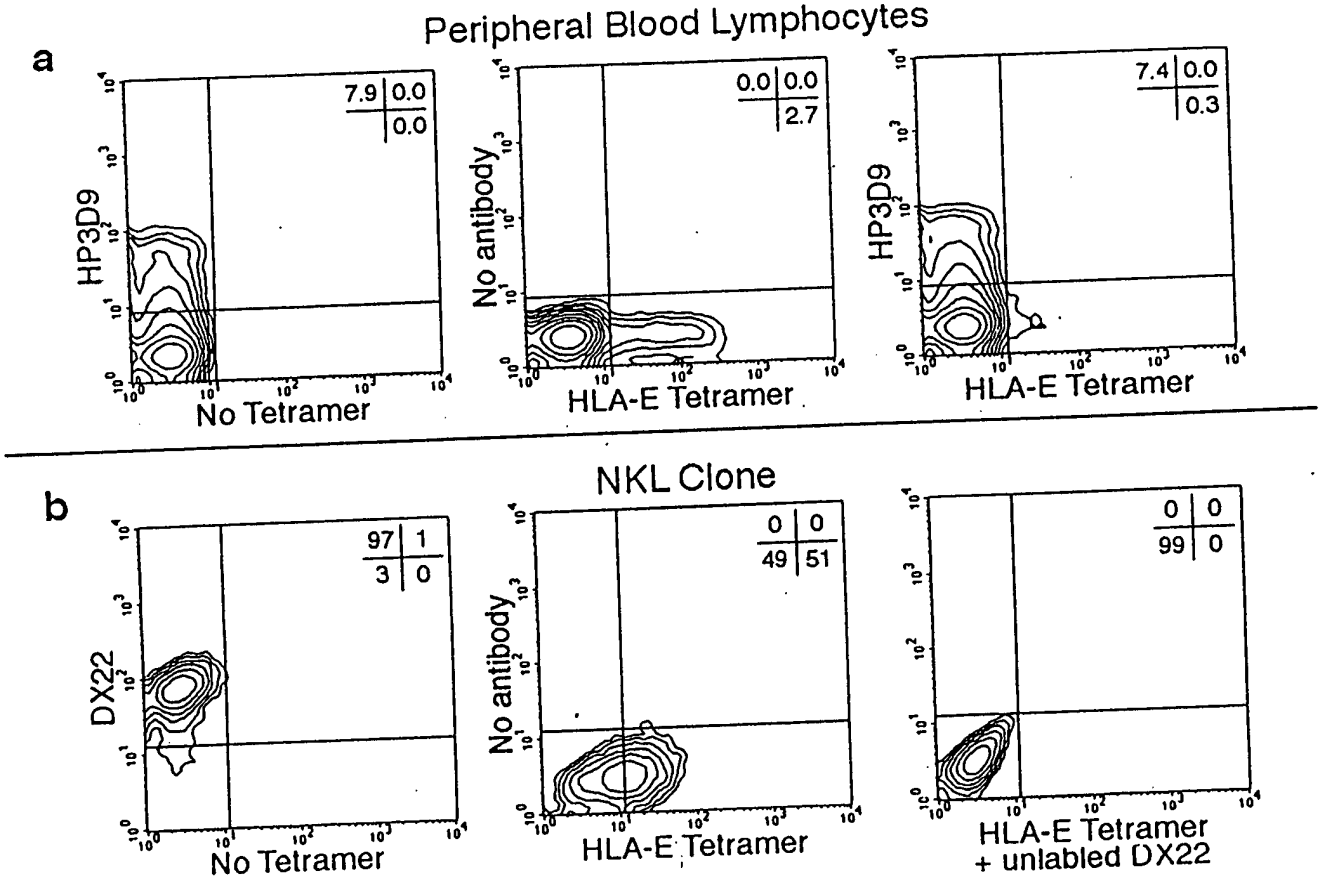

Figure 2

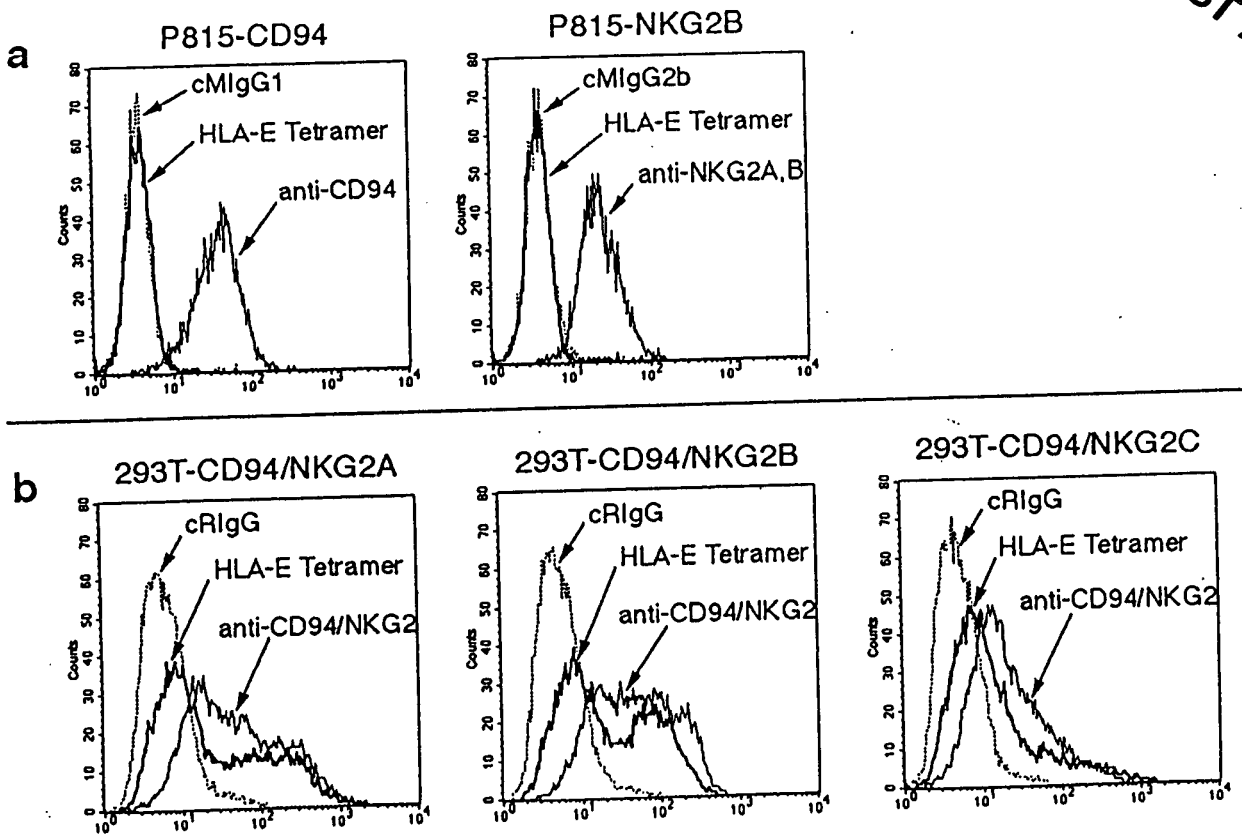

Figure 3